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Overexpression of the Aldose Reductase Gene Induces Apoptosis in Pancreatic β-Cells by Causing a Redox Imbalance[1]

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To determine the role of the polyol metabolizing pathway under hyperglycemic conditions, the effects of aldose reductase (AR) on the cellular functions of pancreatic β-cells were examined. Stable transfectants of rat AR cDNA were obtained with a pancreatic β-cell line, HIT, in which a negligible amount of AR was originally expressed. Overproduction of AR triggered DNA fragmentation, as judged with the TUNEL method and agarose gel electrophoresis. Morphological analysis by electron microscopy also clearly showed apoptosis of the AR-overexpressing HIT cells. Induction by interleukin-1β of gene expression such as those of an inducible form of nitric oxide synthase (NOS-II) and Mn-superoxide dismutase (Mn-SOD), was much lower in the transfectants than in the control cells, while the expression of constitutively expressed genes such as those for Cu,Zn-superoxide dismutase and insulin was not changed. The susceptibility to interleukin-1β stimulation of the expression of the NOS II and Mn-SOD genes was due to suppressed NF-κB activity, which is essential for the expression of these genes. In addition, the intracellular NADPH/NADP+ ratio was considerably lower in the AR-transfected cells than in control cells. Thus, the overexpression of AR in pancreatic β-cells induced apoptosis that may be caused by a redox imbalance.

Key words: aldose reductase, apoptosis, pancreas β-cell, redox imbalance, transfection.

A number of hypotheses including enhancement of the polyol pathway (*1*) and the extent of glycation (*2, 3*), as well as a decrease in the myo-inositol level (*4*) have been proposed to explain the pathogeneses of diabetic complications. Aldose reductase (AR), which reduces glucose to sorbitol with NADPH as the electron donor (*1*), is a key enzyme in the polyol pathway. Sorbitol is subsequently oxidized to fructose by sorbitol dehydrogenase (SDH), a member of the alcohol dehydrogenase gene superfamily, with NAD^+ as the electron acceptor. AR is the rate-determining enzyme in this pathway, with a much higher K_m for glucose than hexokinase, and is relatively inactive under normal conditions. Sorbitol accumulation is induced under hyperglycemic conditions as the result of enhanced AR gene expression and augmented AR activity. The resulting accumulation of sorbitol is thought to play a role in the pathogeneses of diabetic complications (*5-7*).

It is also possible, however, that AR plays a role in pancreatic β-cells. In fact, it has been reported that the function of pancreatic β-cells is impaired by prolonged exposure to a high glucose concentration (*8-10*). Under high glucose conditions, augmented AR activity and resulting activation of the polyol pathway would be expected. The role of AR in the inhibition of insulin secretion is supported, in part, by the following finding: some AR inhibitors improved insulin secretion in diabetic rat pancreatic β-cells (*11*), in which the presence of the polyol pathway has been demonstrated (*12, 13*). However, no appropriate mechanism has yet been reported which explains why the enhanced AR activity under diabetic conditions induces a dysfunction of pancreatic β-cells.

This report describes the examination of the role of AR in the dysfunction of pancreatic β-cells by transfecting rat AR cDNA into HIT cells, in which a negligible amount of AR protein was originally detected. The results clearly show that AR gene-transfected HIT cells exhibit a significant decrease in intracellular NADPH, as well as typical features of apoptosis. This is the first direct demonstration of apoptosis in pancreatic β-cells which has been induced through the overexpression of AR. The relation between the dysfunction of pancreatic β-cells and the high concentration of glucose in the diabetic state is discussed in the

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Abbreviations: AR, aldose reductase; SDH, sorbitol dehydrogenase; SOD, superoxide dismutase; NOS, nitric oxide synthase; GSH, glutathione; GC-MS, gas chromatography-mass spectrometry; PBS, phosphate-buffered saline.

context of the augmentation of AR activity and apoptosis induced by a redox imbalance.

MATERIALS AND METHODS

Materials—[α-^{32}P]dCTP and random hexamer labeling kits were purchased from Amersham. Recombinant human IL-1β was generously provided by Otsuka Pharmaceutical. Leuconostoc glucose-6-phosphate dehydrogenase and beef liver glutamate dehydrogenase were purchased from Sigma Chemical, and 6-phospho-gluconate dehydrogenase from Boehringer Mannheim. Other reagents used were of the highest grade available.

Cell Culture—HIT cells, a hamster-derived β-cell line, were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 U/ml penicillin, and 100 U/ml streptomycin under a humid atmosphere including 5% CO_2 at 37°C.

Probes for Northern Blot Analysis—Total RNAs were isolated from HIT cells by the guanidinium thiocyanate extraction method (*14*). The first cDNA strand was synthesized in a reaction mixture 16 μl, containing 5 μg of total RNA and 0.5 μg of oligo dT primer using a commercial kit according to manufacturer's recommended protocol (Gibco BRL). Specific primers for insulin cDNA were designed and synthesized based on the cDNA sequence of Syrian hamster islet cells (*15*). The nucleotide sequences were 5′-CTAGGTGACCAGCTATAATC-3′ (bases 5-24, sense) and 5′-AGTTGCAGTAGTTCTCTAGC-3′ (bases 349-368, antisense) which amplified a 364 bp product. The *Pst*I fragment (490 bp) of a cloned rat AR cDNA was used as a probe (*16*). To detect SDH mRNA, two oligonucleotide primers, 5′-AAAGCAAGAGAGCGACATGG-3′ and 5′-GGATGGCGTCAGTAAGTATC-3′, were synthesized and used to amplify the cDNA (*17*). PCR was carried out with one-tenth of the cDNA, using 50 pmol of each oligonucleotide primer. The conditions for PCR were 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, for 35 cycles. The products were confirmed by DNA sequence analysis.

Northern Blotting—Subconfluent cells were stimulated with recombinant human IL-1β (5 ng/ml) for 16 h. Total RNA was extracted from the cells, and 20 μg of it was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNA was transferred to a Zeta-Probe membrane (Bio-Rad) by capillary action. After hybridization with a ^{32}P-labeled AR (*16*), SDH, NOS-II (*18*), Mn-SOD (*19*), Cu,Zn-SOD (*19*), or insulin probe at 42°C in the presence of 50% formamide, the membrane was washed at 55°C in 2×SSC (1×SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.5) containing 0.1% sodium dodecyl sulfate for 60 min. Kodak XAR films were exposed for 2 days with an intensifying screen at −80°C.

Transfection of AR cDNA—A full length rat AR cDNA (*16*) was ligated into the *Eco*RI site of pBluescript SK(+) (Stratagene), cut out with *Not*I and *Apa*I, and then ligated into the pRc/CMV expression vector (Invitrogen). The resulting plasmid was purified by CsCl/ethidium bromide equilibrium ultracentrifugation. HIT cells were transfected with 20 μg of the purified plasmid DNA using Lipofectamine reagent (Gibco BRL), and positive clones were selected by treatment with geneticin disulfate (Sigma). pRc/CMV expression vector-transfected cells were used as a control.

SDS-PAGE and Western Blotting—Harvested HIT cells were washed twice with PBS. Cells were resuspended in 100 μl of 20 mM NaH_2PO_4 buffer containing 2 mM $MgCl_2$ (pH 7.0), and then sonicated. The protein concentrations of cell lysates were determined with a BCA kit (Pierce) using bovine serum albumin as a standard. For Western blotting, 10 μg aliquots of cell lysates were electrophoresed on a 12.5% SDS gel and then transferred to a nitrocellulose transfer membrane using Trans-blot (Bio-Rad). After blocking in 4% skimmed milk overnight, the blot was incubated for 2 h at room temperature with 1:1,000 dilutions of an anti-rat AR antibody (*16*) and an anti-rat SDH antibody (*20*). After washing the nitrocellulose membrane three times for 30 min each, the blot was incubated for 2 h with 1:1,000 diluted peroxidase-conjugated anti-rabbit IgG (Cappel) at room temperature. The chemiluminescence method was employed to amplify the signal using an ECL kit (Amersham).

Gel Shift Assay for NF-κB—After incubation of HIT cells with or without IL-1β, nuclear extracts were prepared as described previously (*21*). Six micrograms of a nuclear extract was incubated with 1 μg of poly (dI-dC) in 20 μl of a reaction buffer (10 mM Hepes-KOH, pH 7.8, 50 mM KCl, 5 mM $MgCl_2$, and 10% glycerol) at 4°C. The binding reaction was initiated by the addition of the 5′-end-^{32}P-labeled oligonucleotide for NF-κB binding using gel shift assay systems (Promega). A non-radioactive competitor double-strand oligonucleotide was used to eliminate nonspecific binding. Products were separated by electrophoresis on 5% polyacrylamide gels in 5 mM Tris-HCl, 0.2 mM EDTA, and 76 mM glycine at 100 V for 1 h, and the dried gels were subjected to autoradiography.

Measurement of NADPH/NADP$^+$—NADPH and NADP$^+$ were measured by the enzymatic methods previously described (*22*). Cells were washed twice with PBS and then dissolved in 250 μl of 20 mM Tris-HCl, pH 7.4. After sonication, 5 μl of 5 M HCl was added to the cell lysate and the mixure was boiled for 3 min prior to the measurement of NADP$^+$. To determine NADPH, 5 μl of 5 M NaOH was added to the cell lysate, which was then incubated for 15 min at 60°C prior to boiling. After samples had been neutralized, 5 μl of each samples was reacted with 200 μl of a cycling mixture (100 mM imidazole-HCl, pH 7.4, 5 mM glucose-6-phosphate, 7.5 mM α-keto-glutarate, 25 mM ammonium acetate, 0.1 mM ADP, 0.1% bovine serum albumin, 300 μg/ml beef liver glutamate dehydrogenase, and 10 μg/ml leuconostoc glucose-6-dehydrogenase), followed by incubation at 37°C for 1 h. After boiling for 5 min, 100 μl of each sample was added to the reaction mixture (40 mM imidazole-HCl, pH 7.4, 30 μM NADP$^+$, 2 mM Na_2EDTA, and 30 mM 2 μg/ml 6-phospho-gluconate dehydrogenase) to measure the accumulated 6-phosphogluconate. After incubation for 30 min, resultant NADPH was detected by fluorometrically with excitation at 340 nm and emission at 450 nm.

Detection of Apoptotic Cells by the TUNEL Method—Apoptotic cells were detected by the dideoxynucleotide transferase (TdT)-mediated X-dUTP nick end labeling (TUNEL) method using an *in situ* apoptosis detection kit (Takara Shuzo) according to the manufacturer's recommended protocol. Briefly, cells were cultured on 2 chamber polystyrene vessel tissue culture treated glass slides (Bec-

ton Dickinson), fixed in 4% paraformaldehyde for 20 min at room temperature, and then washed in PBS three times for 5 min each. Endogenous peroxidase was inactivated with 0.3% H_2O_2 in methanol for 15 min. The fixed cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min, and then incubated with fluorescein-labeled dUTP for 60 min at 37°C to detect 3′-OH fragmented DNA ends. After washing in PBS, the cells were incubated with horseradish peroxidase-conjugated anti-fluorescein antibodies for 30 min at 37°C. Apoptotic nuclei were visualized by incubation with a 3,3-diaminobenxidine tetrahydrochloride (Zymed Laboratories) solution for 5 min, and methyl green was used for nuclear counterstaining.

Detection of Apoptotic Cells by Electron Microscopy—Native HIT cells, CMV vector-transfected HIT cells, or AR-transfected HIT cells were cultured in 35 mm plastic dishes, washed three times in PBS, fixed with phosphate-buffered 3% glutaraldehyde (pH 7.4) for 30 min at 4°C, and then further fixed with phosphate-buffered 1% osmium tetraoxide (pH 7.4) for 30 min at 4°C. After washing three times in PBS, the fixed cells were dehydrated with a graded series of ethanol concentrations. These cells were infiltrated with 2-hydroxypropyl methacrylate, and further infiltrated with 50% 2-hydroxypropyl methacrylate in an epoxy resin embedding solution. Cells which had been embedded in the epoxy resin were detached from the plastic dishes. Ultrathin sections obtained with an Ultracut E ultramicrotome (Reihert-Jung) were doubly stained with aqueous uranyl acetate (3%) and Reynold's lead citrate, and then observed under a H-7000 electron microscope (Hitachi).

Detection of DNA Ladder Formation in Apoptotic Cells—For the detection of DNA ladder formation, cells were washed twice with PBS and then sedimented by centrifugation. The cells were dissolved in 230 μl of lysis buffer (20 mM phosphate buffer, pH 7.4, 1% SDS, and 50 μg/ml proteinase K) and then incubated for 90 min at 55°C. The cell lysate was supplemented with 10 μl of 10 mg/ml RNase A and then incubated for 1 h at 37°C, followed by the addition of 300 μl of an NaI solution (6 M NaI, 13 mM EDTA, 10 mg/ml glycogen, 26 mM Tris-HCl, pH 8.0, and 0.5% sodium-*N*-lauroyl sarcosinate) and incubated for a further 15 min at 60°C. The nuclear DNA was precipitated with an equal volume of isopropanol, and then washed with 50% isopropanol and finally with 100% isopropanol. The DNA pellet was dried and dissolved in TE buffer (10 mM Tris-HCl pH 7.5, and 1 mM Na_2EDTA). The resulting DNA was dissolved in TE buffer, extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and then precipitated in 70% (w/v) alcohol with 0.3 M sodium acetate. DNA samples were analyzed by electrophoresis on 1.5% agarose gels, and visualized by staining with a solution containing 1 μg/ml of ethidium bromide.

Evaluation of Sorbitol-Synthesizing Enzyme Activity—Sorbitol-synthesizing enzyme activity was examined as described previously (*23*) with minor modifications. Briefly, 200 μl of a mixture consisting of 15 mM glucose, 1 mM NADPH, 100 mM sodium phosphate buffer, pH 7.4, and the supernatant fraction of the HIT cells was used. After incubation at 37°C under aerobic conditions for 2 h, a known concentration of uniformly ^{13}C-labeled D-sorbitol, which had been synthesized from uniformly ^{13}C-labeled D-glucose (Cambridge Isotope Laboratories), was added to the mixture as an internal standard. After deproteinization with ethanol, the supernatant was evaporated to dryness under reduced pressure. For trifluoroacetate derivatization, pyridine and *N*-methyl-bis-trifluoroacetamide (Pierce Chem.) were added, and the supernatant was analyzed with a G-3000 gas chromatograph (Hitachi) combined with a G-2000 mass spectrometer (Hitachi) on a silicon SE-30 capillary column (30 m × 0.25 mm I.D.; Gasukuro Kogyo). Electron-impact ionization mass spectra were recorded at an ionizing energy of 70 eV and an ionization current of 300 μA. The column temperature was set at 85°C initially, held for 3 min, and then increased to 90°C at the rate of 1°C/min and from 90°C to 220°C at the rate of 40°C/min. The rate of sorbitol synthesis in HIT-cells was obtained after normalization of the data with respect to the cytosolic protein concentration, which was determined with a protein assay kit (Bio-Rad).

RESULTS

Establishment of Stable Lines Overexpressing Rat AR—To elucidate the role of AR in cells, we chose a hamster pancreatic β-cell derived line, HIT cells, which exhibits virtually no AR expression. We established stable transfectants which constitutively expressed rat AR by transfecting AR cDNA under regulation by a CMV promoter and selecting neomycine-resistant clones. Among the four lines isolated, two lines, designated as AR1 and AR2, were found to express relatively large amounts of rat AR, as judged on by Northern and Western blot analyses (Fig. 1). We also examined the expression of SDH, the other enzyme constituting the sorbitol metabolizing pathway, and found slight elevation of the enzyme level in AR1 and AR2 cells.

Sorbitol-synthesizing activity was measured by gas chromatographic-mass spectrometric analysis. The AR transfectants exhibited 2-4-fold increases in activity compared with the controls (Fig. 2). Thus the two clones were found to be lines which stably overproduce rat AR.

Induction of Apoptosis in AR-Transfected Cells—The transfectants exhibited morphological changes, as they were observed to have a round shape compared with the controls (data not shown). Since the viability of these lines was much lower than that of the controls, we examined the characteristics of these lines by several methods. Some

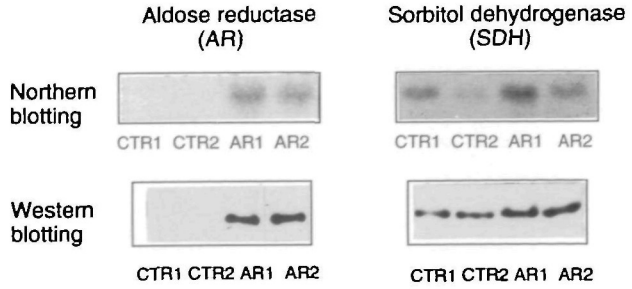

Fig. 1. **Expression of AR and SDH in rat AR cDNA-transfected HIT cells.** 20 μg of total RNA, isolated from cells transfected with the AR gene (AR1 or AR2) or the vector alone (CTL1 or 2), was analyzed by Northern blotting. DNA fragments of rat AR and SDH cDNA sequences were used as probes. 10 μg of cell lysates from transfectants was subjected to Western blot analysis using anti-rat AR and SDH antibodies as the primary antibodies.

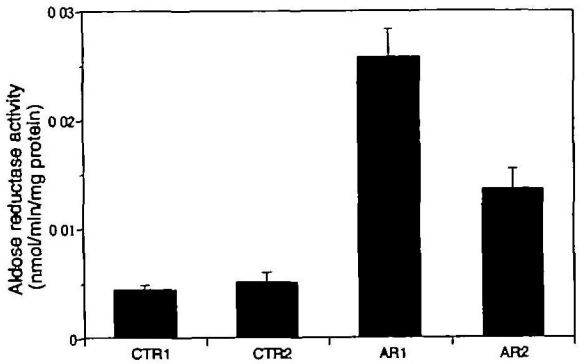

Fig. 2. **Assaying of AR activity by measurement of sorbitol-producing activity in transfectants.** Using trifluoroacetate derivatization and GC-MS method, the sorbitol producing enzyme activities of the transfectants were examined (CTL1, 2, control cells; AR1, 2, AR gene-transfected cells). Data are the means and SD of duplicate assays.

nuclei of cells which had been transfected with AR cDNA were brown in color, and were positive for DNA breakdown, with the TUNEL method (Fig. 3A), and the incidence of such cells was much higher among the transfectants (about 3-5%) than in the controls (less than 0.6%) (Fig. 3B). Fragmentation of DNA was further confirmed by running DNA extracted from the cells on agarose gels (Fig. 4A). AR-transfected cells exhibited notable DNA ladder formation. When the ultrastructure of the cells was microscopically examined, various features of apoptosis, such as chromosomal condensation, formation of apoptotic bodies and fragmentation of cells, were clearly observed in about 5% of the cells (Fig. 4B). Such ultrastructural features were rarely observed in native and CMV-transfected HIT cells. Thus, these data indicate that the decreased viability of the AR-transfected cells was due to apoptosis.

Suppressed Expression of Inducible Genes in AR-Transfected Cells—It has been established that, similar to AR, nitric oxide synthase II (NOS II), which is induced by inflammatory cytokines such as IL-1β and TNF-α in HIT cells (*18*), requires NADPH as a cofactor. To determine the influence of AR overexpression on IL-1β-induced NOS activity, NO_2 production in culture medium was measured by means of the Griess reaction. Because IL-1β-stimulated transfectants produced lower levels of NO_2 than the control cells (data not shown), we examined the expression of the NOS II gene as well as that of the Mn-SOD gene by Northern blot analysis. Although both NOS-II and Mn-SOD mRNAs were induced markedly by IL-1β in control cells, the extent of the induction of these genes was negligible in the AR-transfected cells (Fig. 5A). However, no difference was observed in the expression of constitutive genes, such as those of Cu,Zn-SOD (Fig. 5A), and insulin (Fig. 5B), between the AR-transfected and control cells, indicating that the transcriptional machinery fundamental for gene expression was not affected by AR overexpression.

Defect in DNA Binding of NF-κB—Since the expression of the NOS II and Mn-SOD genes was suppressed, as described above, and their expression is under the control of a transcriptional factor, NF-κB (*24, 25*), we examined whether or not NF-κB is activated in these cells (Fig. 6). After stimulation of the cells with IL-1β for 16 h, nuclear

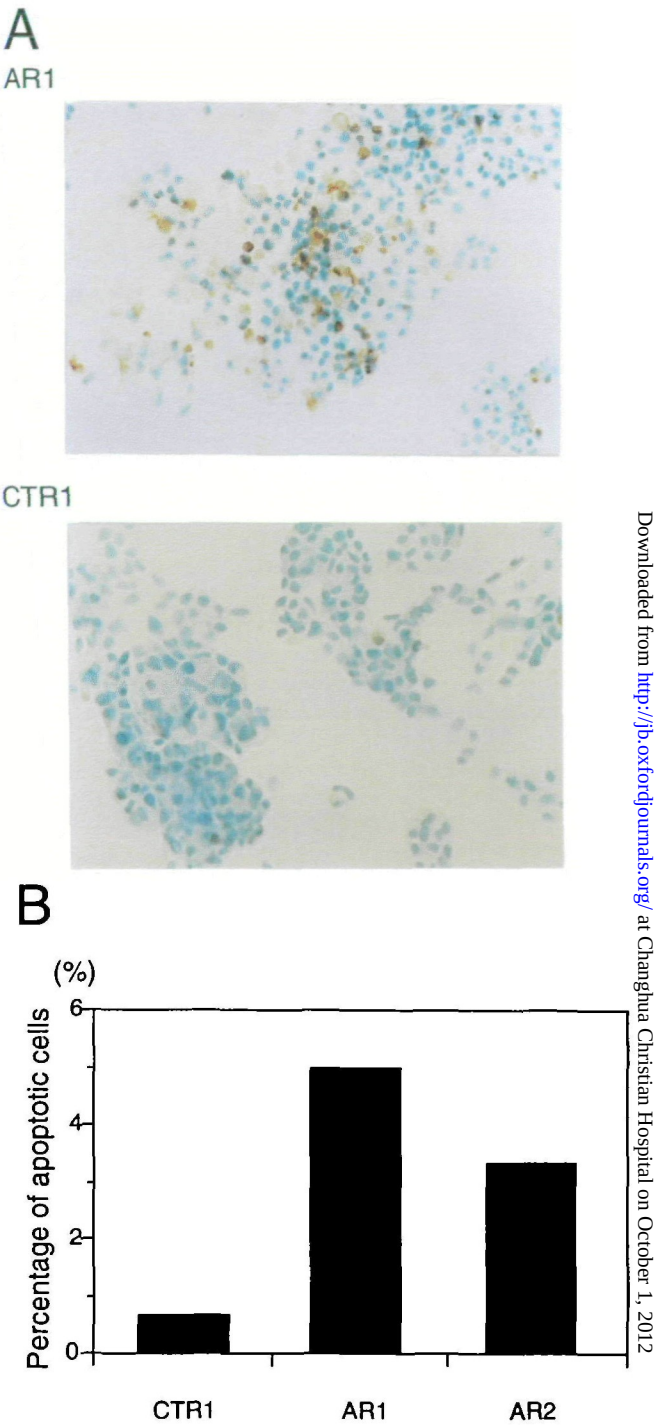

Fig. 3. **Detection of apoptotic cells by the TUNEL method.** (A) Among AR-transfected cells, the nuclei of TUNEL-positive cells undergoing apoptosis were stained brown. Nuclear counterstaining: methyl green. Original magnification ×380. (B) Apoptotic cells with nuclei stained brown among 300 AR-transfected cells, and CMV vector-transfected cells were counted under a light microscope, and their percentages are shown.

extracts were subjected to a gel shift assay involving a NF-κB-specific probe. While strong signals corresponding to the DNA-NF-κB complex were observed for nuclear extracts of control cells, the signals were much weaker for the AR-transfected cells. Thus a reduction of NF-κB activation was evident in the AR-transfected cells, which

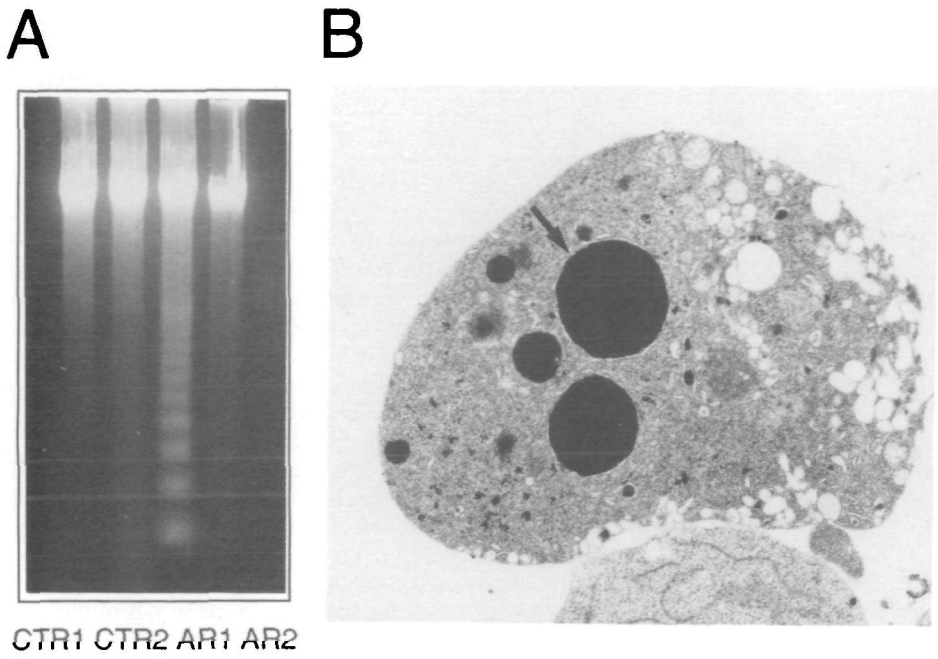

Fig. 4. **Internucleosomal DNA cleavage and morphological changes of the transfectants observed on electron microscopy.** (A) DNA extracted from cells was subjected to 1.5% agarose gel electrophoresis (CTL1, 2, control cells; AR1, 2, AR gene-transfected cells). (B) In AR-transfected HIT cells, a typical ultrastructural feature, electron-dense apoptotic bodies (arrows), was observed. The membrane structures of other organelles were fairly well preserved in these cells, although some parts of the plasma membrane were disrupted. Original magnification, ×7,500.

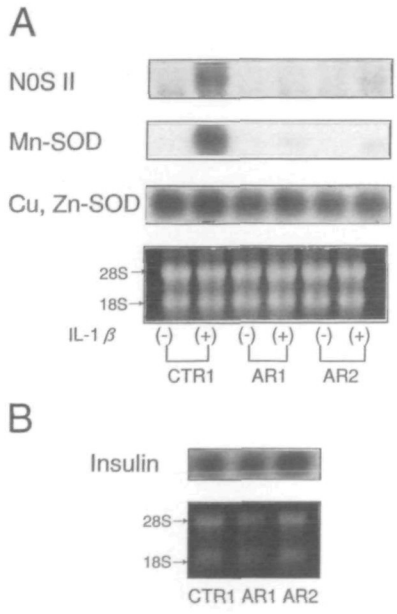

Fig. 5. **Effects of AR overexpression on the expression of some genes in the transfectants.** (A) Cells were cultured in the presence (+) or absence (−) of 5 ng/ml IL-1β for 16 h. 20 μg of total RNA was isolated, and then subjected to Northern blotting with NOS II, Mn-SOD, Cu,Zn-SOD (A), or insulin (B) cDNA as a probe. The positions of 18S and 28S ribosomal RNA are indicated (CTL1, control cells; AR1, 2, AR gene-transfected cells).

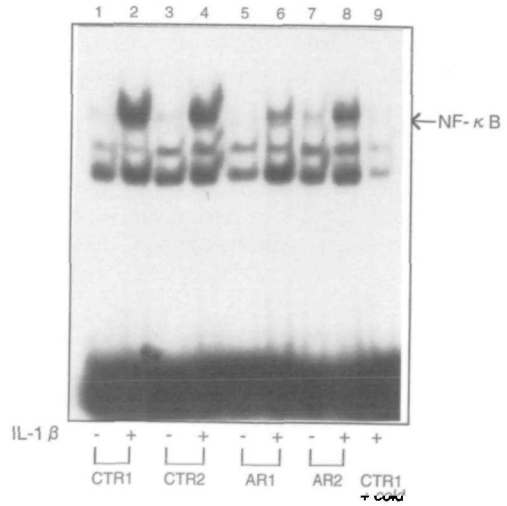

Fig. 6. **Effect of AR overexpression on IL-1β-induced activation of NF-κB.** The lanes contain nuclear extracts of transfectants incubated with (+) or without (−) 5 ng/ml IL-1β for 16 h. 6 μg of each nuclear extract was used to determine the activation of NF-κB by means of a gel shift assay involving oligonucleotides specific for NF-κB binding. All lanes contained the labeled NF-κB probe. Lane 9 contained a 100-fold excess of the cold oligonucleotides in addition to the other components.

represents a likely cause of the weak induction of expression of the NOS II and Mn-SOD genes.

Redox Imbalance in AR-Transfected Cells—Since AR consumes NADPH during the catalytic conversion of glucose to sorbitol, and the redox potential is largely dependent on the balances between NADPH/$NADP^+$ and NAD^+/NADH in cells, we measured the intracellular contents of NADPH/$NADP^+$ (Table I). Although the sum of

TABLE I. **Levels of NADPH and $NADP^+$ in transfectants and control cells.** The means and SD of triplicate determinations are shown.

	NADPH (nmol/g)	$NADP^+$ (nmol/g)	NADPH/$NADP^+$
CTR1	74.4±1.6	62.5±1.2	1.19
AR1	40.1±4.5	73.4±0.6	0.55
AR2	63.7±0.9	99.2±0.5	0.64

$NADP^+$ and NADPH was approximately the same, the ratios were significantly lower in AR-transfected cells,

compared to in control cells. Thus the AR-transfected cells had a lower redox potential than the control cells.

DISCUSSION

Insulin-independency of the glucose uptake in organs such as the lens and kidney, and neurons is postulated to be an important factor in the development of diabetic complications. Glucose is freely permeable in the lens, and an increase in the free intracellular glucose level which is sufficient for saturation or exceeds that required for hexokinase activity is frequently observed under diabetic conditions. Consequently, in these organs, AR activity is augmented, and sorbitol and fructose are generated. Transgenic mice expressing human AR show elevation of the sorbitol concentration in liver, kidney and muscle on injection of a 30% glucose diet for 5 days (*26*). In pancreatic β-cells, glucose uptake is mediated by high-K_m glucose-transporter 2 and increases in proportion to an increase in the extracellular glucose concentration (*27*). Under hyperglycemic conditions, additional glucose is thus readily available for metabolism through the polyol pathway, which generates sorbitol and fructose. The AR-transfected HIT cells described here provide a model for pancreatic β-cells which show enhanced metabolic activity in the polyol pathway. As a result, when AR is overexpressed, HIT cells exhibit typical features of apoptosis.

Considering the mechanism of the apoptosis, which is induced in AR-transfected HIT cells, the decrease in intracellular NADPH and the defect in the activation of NF-$\varkappa$B are particularly noteworthy. NADPH is required as a cofactor for the intracellular redox regulation of compounds such as thioredoxin reductase and glutathione reductase. Thioredoxin reductase, an NADPH-dependent seleno-flavoprotein, reduces the active sites of cysteine residues of thioredoxin (TRX), which is a low molecular mass redox (reduction/oxidation) active protein (*28*). TRX is also involved in intracellular signaling related to the growth and apoptosis of cells *via* the regulation of transcription factors such as NF-$\varkappa$B. Under unstimulated conditions in the cytosol, NF-$\varkappa$B is associated with an inhibitory molecule, I$\varkappa$-B, and cannot bind DNA (*29*). Activation of cells with appropriate stimuli results in the dissociation of NF-$\varkappa$B from I$\varkappa$-B. NF-$\varkappa$B is then restored by TRX reductase through a thiol-disulfide exchange reaction through cysteine residues, and is subsequently translocated into nuclei (*30*). In AR-overexpressing HIT cells, the amount of nuclear NF-$\varkappa$B was markedly decreased. This may be due to a decrease in the level of the reduced form of TRX by virtue of a redox imbalance. Moreover, in mammalian cells the reduced form of TRX is a physiological inhibitor of apoptosis signal-regulating kinase (ASK-1), which was recently identified as a mitogen-activated protein (MAP) kinase kinase kinase which activates c-Jun N terminal kinase (JNK) and p38 MAP kinase (*31*). In this scheme, the reduced form of TRX binds directly to ASK-1, thus inhibiting ASK-1 kinase activity (*32*). Taken together NADPH consumption elicited by AR overexpression and the redox imbalance may result in a decrease in the level of the reduced form of TRX, apoptosis of HIT cells thereby being induced.

As an alternative intracellular redox regulatory molecule, reduced glutathione (GSH) exists at millimolar levels in most cells, functions as an antioxidant, and also acts as a cofactor for some enzymes involved in antioxidation, and redox regulation. The level of GSH is decreased and the ratio of NADPH/$NADP^+$ is lower in diabetic patients, and an inhibitor of AR partially restores them (*33*). However, it is well known that the levels of antioxidants (*34*) including GSH (*35*) are low in pancreatic β-cells. Thus, the effect of the redox imbalance reflected by the decrease of NADPH in AR-overexpressing HIT cells may be unable to be compensated for by the GSH system. Along with this, low levels of antioxidants in pancreatic β-cells may explain why β-cells are so susceptible to oxidative stress due to compounds such as streptozotocin and nitric oxide (*18*), while overexpression of antioxidative molecules such as Cu,Zn-SOD (*36*) and TRX (*37*) has a protective effect against oxidative reagent-induced diabetes.

The precise molecular mechanism by which apoptosis occurs in AR-overexpressing HIT cells awaits further investigation. The present study, however, provides the first demonstration of apoptosis in pancreatic β-cells induced by AR-overexpression. The present AR-transfectants may be useful for identifying novel AR inhibitors which are particularly effective for protecting pancreatic β-cells under diabetic conditions.

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REFERENCES

1. Clements, R., Jr. (1986) The polyol pathway. A historical review. *Drugs* **2**, 3-5
2. Brownlee, M. (1995) Advanced protein glycosylation in diabetes and aging. *Annu. Rev. Med.* **46**, 223-234
3. Vlassara, H. (1997) Recent progress in advanced glycation end products and diabetic complications. *Diabetes* **46**, S19-25
4. Palmano, K.P., Whiting, P.H., and Hawthorne, J.N. (1997) Free and lipid myo-inositol in tissues from rats with acute and less severe streptozotocin-induced diabetes. *Biochem. J.* **167**, 229-235
5. Cohen, M.P. (1986) Aldose reductase glomerular metabolism and diabetic nephropathy. *Metabolism* **35**, 55-59
6. Malone, J.I., Lowitt, S., and Cook, W.R. (1990) Nonosmotic diabetic cataracts. *Pediatr. Res.* **27**, 293-296
7. Pfeifer, M.A. and Schumer, M.P. (1995) Clinical trials of diabetic neuropathy: past, present, and future. *Diabetes* **44**, 1335-1361
8. Poitout, V. and Robertson, R.P. (1996) An integrated view of beta-cell dysfunction in type-II diabetes. *Annu. Rev. Med.* **47** 69-83
9. Unger, R.H. and Grundy, S. (1985) Hyperglycaemia as an inducer as well as a consequence of impaired islet cell function and insulin resistance implications for the management of diabetes. *Diabetologia* **28**, 119-121
10. Leahy, J.L., Bonner-Weir, S., and Weir, G.C. (1992) β-cell dysfunction induced by chronic hyperglycemia. Current ideas on mechanism of impaired glucose-induced insulin secretion. *Diabetes Care* **15**, 442-455
11. Nakayama, K., Murakami, N., Ohta, M., Kato, K., Notsu, T., Mizota, M., Miwa, I., and Okuda, J. (1995) Effects of M16209 on insulin secretion in isolated, perfused pancreases of normal and diabetic rats. *Eur. J. Pharmacol.* **276**, 85-91
12. Morrison, A.D., Winegrad, A.I., Fink, C.J., and Lacy, P.E. (1970) Sorbitol synthesis in isolated rat pancreatic islets. *Biochem. Biophys. Res. Commun.* **38**, 491-495
13. Dufrane, S.P., Malaisse, W.J., and Sener, A. (1984) A micromethod for the assay of aldose reductase, its application to pancreatic islets. *Biochem. Med.* **32**, 99-105

14. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
15. Bell, G.I. and Sanchez-Pescador, R. (1984) Sequence of a cDNA encoding Syrian hamster preproinsulin. *Diabetes* **33**, 297-300
16. Takahashi, M., Fujii, J., Miyoshi, E., Hoshi, A., and Taniguchi, N. (1995) Elevation of aldose reductase gene expression in rat primary hepatoma and hepatoma cell lines: implication in detoxification of cytotoxic aldehydes. *Int. J. Cancer* **62**, 749-754
17. Karlsson, C., Jornvall, H., and Hoog, J.O. (1991) Sorbitol dehydrogenase: cDNA coding for the rat enzyme. Variations within the alcohol dehydrogenase family independent of quaternary structure and metal content. *Eur. J. Biochem.* **198**, 761-765
18. Kaneto, H., Fujii, J., Seo, H.G., Suzuki, K., Matsuoka, T., Nakamura, M., Tatsumi, H., Yamasaki, Y., Kamada, T., and Taniguchi, N. (1995) Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes* **44**, 733-738
19. Fujii, J. and Taniguchi, N. (1991) Phorbol ester induces manganese-superoxide dismutase in tumor necrosis factor-resistant cells. *J. Biol. Chem.* **266**, 23142-23146
20. Hoshi, A., Takahashi, M., Fujii, J., Myint, T., Kaneto, H., Suzuki, K., Yamasaki, Y., Kamada, T., and Taniguchi, N. (1996) Glycation and inactivation of sorbitol dehydrogenase in normal and diabetic rats. *Biochem. J.* **318**, 119-123
21. Sadowski, H.B. and Gilman, M.Z. (1993) Cell-free activation of a DNA-binding protein by epidermal growth factor. *Nature* **362**, 79-82
22. Kato, T., Berger, S.J., Carter, J.A., and Lowry, O.H. (1973) An enzymatic cycling method for nicotinamide-adenine dinucleotide with malic and alcohol dehydrogenases. *Anal. Biochem.* **53**, 86-97
23. Kishimoto, M., Kajimoto, Y., Kubota, M., Watarai, T., Shiba, Y., Kawamori, R., Inaba, T., Kamada, T., and Yamasaki, Y. (1997) Sensitive, selective gas chromatographic-mass spectrometric analysis with trifluoroacetyl derivatives and a stable isotope for studying tissue sorbitol-producing activity. *J. Chromatogr. B Biomed. Sci. Appl.* **688**, 1-10
24. Xie, Q.W., Kashiwabara, Y., and Nathan, C. (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705-4708
25. Das, K.C., Lewis-Molock, Y., and White, C.W. (1995) Activation of NF-κB and elevation of MnSOD gene expression by thiol reducing agents in lung adenocarcinoma (A549) cells. *Am. J. Physiol.* **269**, L588-602
26. Yamaoka, T., Nishimura, C., Yamashita, K., Itamura, M., Yamada, T., Fujimoto, J., and Kokai, Y. (1995) Acute onset of diabetic pathological changes in transgenic mice with human aldose reductase cDNA. *Diabetologia* **38**, 255-261
27. Johnson, J.H., Newgard, C.B., Milburn, J.L., Lodish, H.F., and Thorens, B. (1990) The high K_m glucose transporter of islets of Langerhans is functionally similar to the low affinity transporter of liver and has an identical primary sequence. *J. Biol. Chem.* **265**, 6548-6551
28. Luthman, M. and Holmgren, A. (1982) Rat liver thioredoxin and thioredoxin reductase: purification and characterization. *Biochemistry* **21**, 6628-6633
29. Schreck, R., Rieber, P., and Baeuerle, P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.* **10**, 2247-2258
30. Hayashi, T., Ueno, Y., and Okamoto, T. (1993) Oxidoreductive regulation of nuclear factor κB. Involvement of a cellular reducing catalyst thioredoxin. *J. Biol. Chem.* **268**, 11380-11388
31. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**, 90-94
32. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobimume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.* **17**, 2596-2606
33. Bravi, M.C., Pietrangeli, P., Laurenti, O., Basili, S., Cassone-Faldetta, M., Ferri, C., and De Mattia, G. (1997) Polyol pathway activation and glutathione redox status in non-insulin-dependent diabetic patients. *Metabolism* **46**, 1194-1198
34. Lenzen, S., Drinkgern, J., and Tiege, M. (1996) Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic. Biol. Med.* **20**, 463-466
35. Wohaieb, S.A. and Godin, D.V. (1987) Alterations in tissue antioxidant systems in the spontaneously diabetic (BB Wistar) rat. *Diabetes* **36**, 1014-1017
36. Kubisch, H.M., Wang, J., Bray, T.M., and Phillips, J.P. (1997) Targeted overexpression of Cu/Zn superoxide dismutase protects pancreatic β-cells against oxidative stress. *Diabetes* **46**, 1563-1566
37. Hotta, M., Tashiro, F., Ikegami, H., Niwa, H., Ogihara, T., Yodoi, J., and Miyazaki, J. (1998) Pancreatic β cell-specific expression of thioredoxin, an antioxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. *J. Exp. Med.* **188**, 1445-1451